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Na/K-ATPase Tethers Phospholipase C and IP3 Receptor into a Calcium-regulatory Complex

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We have shown that the caveolar Na/K-ATPase transmits ouabain signals via multiple signalplexes. To obtain the information on the composition of such complexes, we separated the Na/K-ATPase from the outer medulla of rat kidney into two different fractions by detergent treatment and density gradient centrifugation. Analysis of the light fraction indicated that both PLC-γ1 and IP3 receptors (isoforms 2 and 3, IP3R2 and IP3R3) were coenriched with the Na/K-ATPase, caveolin-1 and Src. GST pulldown assays revealed that the central loop of the Na/K-ATPase α1 subunit interacts with PLC-γ1, whereas the N-terminus binds IP3R2 and IP3R3, suggesting that the signaling Na/K-ATPase may tether PLC-γ1 and IP3 receptors together to form a Ca^{2+}-regulatory complex. This notion is supported by the following findings. First, both PLC-γ1 and IP3R2 coimmunoprecipitated with the Na/K-ATPase and ouabain increased this interaction in a dose- and time-dependent manner in LLC-PK1 cells. Depletion of cholesterol abolished the effects of ouabain on this interaction. Second, ouabain induced phosphorylation of PLC-γ1 at Tyr^{783} and activated PLC-γ1 in a Src-dependent manner, resulting in increased hydrolysis of PIP2. It also stimulated Src-dependent tyrosine phosphorylation of the IP3R2. Finally, ouabain induced Ca^{2+} release from the intracellular stores via the activation of IP3 receptors in LLC-PK1 cells. This effect required the ouabain-induced activation of PLC-γ1. Inhibition of Src or depletion of cholesterol also abolished the effect of ouabain on intracellular Ca^{2+}.

INTRODUCTION

The Na/K-ATPase, or sodium pump, is a ubiquitous transmembrane enzyme that transports Na^+ and K^+ across the plasma membrane by hydrolyzing ATP (Skou, 1988; Lingrel and Kuntzweiler, 1994). Recent work from several laboratories indicates that the enzyme also functions as a signal-transducing receptor for both endogenous and exogenous cardiotonic steroids such as ouabain (Kometiani *et al.*, 1998; Xie *et al.*, 1999; Haas *et al.*, 2000, 2002; Liu *et al.*, 2000; Aizman *et al.*, 2001; Aydemir-Koksoy *et al.*, 2001; Miyakawa-Naito *et al.*, 2003). First, binding of ouabain to the signaling Na/K-ATPase activates multiple signaling pathways and regulates transcription and translation of many genes in cardiac myocytes and other cell types. Second, activation of several of these pathways is independent of changes in intracellular ion concentrations (Liu *et al.*, 2000; Aydemir-Koksoy *et al.*, 2001; Miyakawa-Naito *et al.*, 2003). Third, Na/K-ATPase is found to interact directly with neighboring membrane proteins and organized cytosolic cascades of signaling complexes to transmit the ouabain signal to different intracellular compartments (Xie and Cai, 2003). Specifically, we have shown recently that the Na/K-ATPase is concentrated in caveolae together with its signaling partners and that binding of ouabain to the caveolar Na/K-ATPase activated the Na/K-ATPase signaling complex, resulting in tyrosine phosphorylation of multiple proteins including epidermal growth factor receptor (EGFR; Wang *et al.*, 2004). Finally, like other receptors, activation of the signaling function of the Na/K-ATPase by ouabain induces the endocytosis of the enzyme (Liu *et al.*, 2004).

It is well known that ouabain regulates $[Ca^{2+}]_i$ via the Na/K-ATPase in cardiac myocytes (Kelly and Smith, 1993). Several years ago, we showed that ouabain-induced increases in $[Ca^{2+}]_i$ required the activation of the signaling function of the enzyme because inhibition of Src or ERKs was able to block the effects of ouabain on $[Ca^{2+}]_i$ in cardiac myocytes (Tian *et al.*, 2001). Recently, ouabain was also found to evoke calcium oscillations in renal epithelial cells as well as endothelial cells independent of changes in intracellular Na^+ concentration (Aizman *et al.*, 2001; Saunders and Scheiner-Bobis, 2004). In view of these recent advances, we tested the hypotheses that the Na/K-ATPase has scaffolding function, capable of assembling a calcium-regulatory complex and that ouabain regulates the function of this complex by activation of protein tyrosine kinases. Pig LLC-PK1 cells were chosen for this work because we have previously used these cells as a model to dissect how the Na/K-ATPase is

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Abbreviations used: EGFR, EGF receptor; GST, glutathione *S*-transferase; RTK, receptor tyrosine kinases; ERK, extracellular signal-regulated kinase; RKE, rat kidney Na/K-ATPase; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; RIPA, radioimmunoprecipitation buffer; $[Ca^{2+}]_i$, intracellular calcium; IP, immunoprecipitation; IB, immunoblotting; PIP2, phosphatidylinositol-4,5-bisphosphate; IP3, inositol 1,4,5-trisphosphate; IP3R2, IP3 receptor isoform 2; DAG, 1,2-diacylglycerol; PLC, phospholipase C; GFP, green fluorescence protein; PH domain, pleckstrin homology domain; ER, endoplasmic reticulum; PMSF, phenylmethanesulfonyl fluoride; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine; Mβ-CD, methyl-β-cyclodextrin.

involved in signal transduction (Liu *et al.*, 2004; Wang *et al.*, 2004). These cells were derived from renal proximal tubules and only express ouabain-sensitive α1 isoform of Na/K-ATPase.

MATERIALS AND METHODS

Materials

The antibodies used and their sources are as follows: The anti-Src monoclonal antibody (mAb), anti-PLC-γ1 mAb, anti-pY^{783}-PLC-γ1 goat polyclonal antibody, anti-phosphotyrosine (PY99) mAb, anti-ERK rabbit polyclonal antibody, anti-caveolin-1 rabbit polyclonal antibody, and anti-IP3R2 goat polyclonal antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-IP3R1 rabbit polyclonal antibody was purchased from Abcam (Cambridge, United Kingdom). The anti-IP3R3 mAb was purchased from BD Transduction Laboratories (San Diego, CA). The monoclonal anti-Na/K-ATPase α1 antibody (α6F) was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). The monoclonal anti-β1 antibody, rabbit polyclonal anti-Na/K-ATPase α1 antibody, rabbit polyclonal anti-phosphotyrosine antibody, protein G and A Agarose, recombinant Src, Src kinase buffer were obtained from Upstate Biotechnology (Lake Placid, NY). The sequencing grade modified trypsin (V5113) was purchased from Promega (Madison, WI). Plasmid containing enhanced GFP (EGFP)-tagged PH domain of PLC-δ1 (PH-GFP) was a kind gift from Dr. David I. Yule (University of Rochester Medical Center, Rochester, NY). Inhibitors PP2 and Xestospongin C were obtained from Calbiochem (La Jolla, CA); and U73122 was obtained from Biomol Research Laboratories (Plymouth Meeting, PA). All secondary antibodies were conjugated to horseradish peroxidase; therefore, the immunoreactive bands were developed using a Western Lightning Chemiluminescence kit (PerkinElmer Life Science, Boston, MA). The Optitran nitrocellulose membranes used for Western blotting were obtained from Schleicher & Schuell BioScience (Keene, NH).

Cell Preparation and Culture

Pig LLC-PK1 cells, mouse SYF and SYF+Src cells were obtained from American Type Culture Collection and were cultured in DMEM medium containing 10% fetal bovine serum (FBS), and penicillin (100 U/ml)/streptomycin (100 μg/ml) as we previously described (Wang *et al.*, 2004). When cell cultures reached ~80% confluence, LLC-PK1 cells were serum-starved for 24 h and used for the experiments.

Preparation of Caveolin-associated Na/K-ATPase from Rat Kidney Outer Medulla

Rat kidney Na/K-ATPase (RKE) was partially purified and separated into two fractions with significantly different contents of caveolin-1 by the procedure we have previously used for the separation of such fractions from pig kidney (Ivanov and Askari, 2004). In brief, male Sprague-Dawley rats weighing between 250 and 300 g were anesthetized with pentobarbital sodium and the kidneys were rapidly collected. The pink-colored outer medulla from 20 rat kidneys was removed and homogenized in buffer A (300 mM sucrose, 30 mM histidine, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.4). Microsomal membranes were prepared from this homogenate and treated with low SDS concentration according to Jorgensen (Jorgensen, 1988) under conditions that do not solubilize Na/K-ATPase but remove impurities from microsomal membranes. The SDS-treated microsomes were then added to the centrifuge tubes as follows: 4.0 ml of 64% glycerol in imidazole buffer (25 mM imidazole, 1 mM EDTA, pH 7.4), 10 ml of 44% glycerol in imidazole buffer, 10 ml of SDS-treated microsomes in 12% sucrose, and 12 ml of imidazole buffer; and centrifuged at 49,000 rpm at 4°C for 3 h in a Beckman type 60Ti rotor (Fullerton, CA). After centrifugation and removal of 1.5 ml of the solution from the bottom, 4.0 ml of heavy fraction containing noncaveolar Na/K-ATPase was collected from the bottom of the centrifuge tube. Afterward, we removed additional 6.0 ml solution from the bottom and collected the light fraction (4.0 ml). Both heavy and light fractions were then centrifuged at 60,000 rpm in a Beckman type 65 rotor at 4°C for 90 min. The pellets were resuspended in 500-1000 μl of buffer A and protein assay was performed. Control fractionation experiments showed that both heavy and light fractions contained enriched Na/K-ATPase (Figure 1). The enzyme collected from the heavy fraction represented the classical Jorgensen preparation (Jorgensen, 1988) and showed the highest specific activity. The enzyme collected from the light fraction represented the caveolar Na/K-ATPase (Figure 1 and Ivanov and Askari, 2004). Specific Na/K-ATPase activities were ~350 and 242 μmol Pi/h/mg protein for the heavy and light fractions, respectively. Specific activity of the microsomal preparation was 52 μmol Pi/h/mg protein. Although a sevenfold increase in activity relative to the microsomal preparation was achieved in our preparation (heavy fraction), it is lower than that of the pig preparation, which usually resulted in >10-fold enrichment of the Na/K-ATPase.

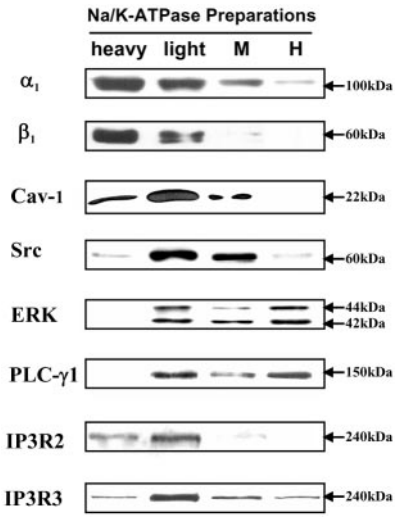

Figure 1. Western blot analysis of the proteins that are coenriched with the Na/K-ATPase. An aliquot of protein (5 μg) was loaded on each lane and blotted against the specific antibodies as indicated (heavy, heavy fraction; light, light fraction, see *Materials and Methods* for details; H, homogenate; M, microsome). Cav-1, caveolin-1.

In Gel Trypsin-digestion and MALDI-TOF Analysis

Sample preparation for MALDI-TOF MS analysis was performed according to the protocol described by Pandey (Pandey *et al.*, 2000). The light fraction of Na/K-ATPase (10 μg) was separated on 10% SDS-PAGE and the gel was silver-stained. After rinsing with water, the bands of interest were excised, minced into 1 × 1-mm pieces and subjected to trypsin digestion as described previously (Pandey *et al.*, 2000). The masses of the resulting peptides were determined using MALDI-TOF MS analysis performed by Protein Structure Facility of University of Michigan (Ann Arbor, MI) and the peptide mass data were analyzed using MS-Fit from Mass Spectrometry Facility of UCSF (Chamrad *et al.*, 2004).

Immunoprecipitation and GST Pulldown Assay

Cells were lysed in RIPA buffer as described previously (Haas *et al.*, 2000). Lysates from LLC-PK1 cells, or other identified cell types, were cleared by centrifugation at 16,000 × *g* for 15 min at 4°C, and the supernatants (1 mg) were either immunoprecipitated using various antibodies or incubated with different GST fusion proteins (Wang *et al.*, 2004). The immunoprecipitates or GST pulldown products were dissolved in sample buffer, separated on 7% SDS-PAGE, transferred to an Optitran membrane and probed with a monoclonal anti-Na/K-ATPase α1, or anti-IP3R2, or anti-PLC-γ1 antibodies. GST-NT (amino acid residue 6–90) and GST-CD3 (amino acid residue 350–785) expression vectors were constructed based on the sequence of the canine Na/K-ATPase α1 subunit. All constructs were verified by DNA sequencing. GST and GST fusion proteins were expressed in *Escherichia coli*, and purified on glutathione Sepharose 4B (Amersham Biosciences, Piscataway, NJ) beads. To immunoprecipitate tyrosine phosphorylated proteins, the cleared cell lysates were incubated with an anti-phosphotyrosine antibody and the immunoprecipitates were analyzed by Western blot using anti-IP3R2 or anti-PLC-γ1 antibody.

Measurement of Intracellular Ca^{2+} Concentration

$[Ca^{2+}]_i$ was measured as previously described (Giovannucci *et al.*, 2000). In brief, serum-starved LLC-PK1 cells were incubated with 2 μM Fura-2/AM at 25°C for 30 min in a physiological salt solution containing: 100 mM NaCl, 4 mM KCl, 20 mM HEPES, 25 mM $NaHCO_3$, 1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1 mM NaH_2PO_4, and 10 mM D-glucose. Ratiometric imaging was performed using a chamber mounted on the stage of a Nikon TE2000-S microscope equipped with a Nikon Super Fluo 40×/NA = 1.30 epifluorescence oil-immersion objective (Melville, NY). Fura-2-loaded cells were locally superfused at a rate of 1 ml/min with the above physiological salt solution. $[Ca^{2+}]_i$ imaging was performed using TILL-Photonics Polychrome IV digital fluorescence imaging system. Fura-2-loaded cells were alternately excited at wavelength of 340 or 380 ± 15 nm, and emission fluorescence was collected with a 510 ± 25 nm bandpass filter (Chroma, Rockingham, VT). Cells were excited for 2 ms every 5 s and monitored for 30 min. Collected images were analyzed using VISION software (New Milford, CT) from TILL-Photonics (Martinsreid, Germany).

Table 1. MS-identification of IP3 receptor (isoform 2)

m/z Submitted	MH^+ matched	Delta Da	Modifications	Start	End	Database sequence
934.2700	933.4179	0.85		562	568	(R) HSQQDYR (K)
974.4200	974.5159	−0.01		334	342	(R) DGELPTSKK (K)
980.4100	979.5511	0.86		555	561	(R) LCYRVLR (H)
1012.3900	1012.4859	−0.01	1Cys-am	1199	1205	(K) CRNQHQR(L)
1246.7900	1247.6860	−0.90		1248	1258	(R) GNPQNQVLLHK (H)
1261.6800	1261.6866	0.00	1Met-ox	989	998	(R) ISYMLSIYKK(E)
1294.7900	1294.7119	0.078		91	101	(K) KLQHAAELEQK (Q)
1332.9000	1332.6106	0.29		1838	1849	(R) EEDSDLMALGPR (M)
1451.1400	1451.6767	−0.54		1723	1737	(K) TAQVGGGFTGQDADK (T)
1506.3200	1506.7561	−0.44		1649	1661	(R) CGAFMSKLINHTK (K)
1528.1600	1527.7300	0.43	2Cys-am	2560	2571	(K) TTCFICGLERDK(F)
1837.7700	1837.8610	−0.1	3Met-ox	920	935	(R) TIHGVGEMMTQMVLSR(G)
1869.6500	1868.9587	0.69		870	884	(R) NLIYFGFYSFSELLR (L)
2115.2000	2115.1306	0.069		1905	1923	(K) SAEEVTMSPAITIMRPILR (F)
2309.4200	2309.9212	−0.50		1884	1904	(R) EMDPDIDTMCPGQEAGSAEEK (S)
2552.7200	2552.2893	0.43	1Cys-am	2157	2177	(R) TMEQIVFPVPNICEFLTRESK(Y)

Imaging Analysis of the Translocation of PLC-δ1-PH-GFP

To monitor PIP2 hydrolysis and IP3 production in live LLC-PK1 cells, we used a recently developed protocol using GFP-fused PLC-δ1 PH domain as described previously (Hirose *et al.*, 1999; Isshiki *et al.*, 2004). LLC-PK1 cells were seeded in six-well plates at ~25,000 cells per well on 25-mm glass coverslips and cultured in 2 ml DMEM supplemented with 10% serum. PH-GFP was transfected into cells in Opti-MEM medium using Lipofectamine 2000 at 1 μg DNA/well. After 24 h, cells were used for the experiments. For imaging, coverslips with cells were mounted on an inverted Leica DM IRE2 microscope and superfused with the physiological salt solution kept at 37°C in a water bath. Confocal imaging was obtained by using a Leica inverted microscope fitted with TCS-SP2 scanhead (Leica, Mannheim, Germany). Excitation of GFP was achieved by using the 488 nm laser-line, and emission was collected at 500–560 nm. For time-lapse studies, imaging areas along the plasma membrane and corresponding cytosolic region were chosen randomly and a series of confocal images were taken at 5-s intervals. Visualization and analysis was performed using Leica Confocal Software.

In Vitro Tyrosine Phosphorylation Assay

The assay was performed according to the protocol described previously (Jayaraman *et al.*, 1996). Briefly, cell lysates were immunoprecipitated using a polyclonal anti-IP3R2 antibody. The immunoprecipitates were washed twice with phosphate-buffered saline (PBS) and suspended in the Src kinase buffer (Upstate Biotechnology). Protein phosphorylation was started by addition of Mg^{2+}/ATP (50 mM/50 μM) alone or with 3 U of recombinant Src. The reactions continued for 5 min at 30°C and were stopped by addition of 2× Laemmli sample buffer. Samples were then separated on SDS-PAGE and analyzed by Western blot using anti-phosphotyrosine (PY99) antibody.

Cholesterol Depletion

Cholesterol depletion was carried out by incubating the cells in DMEM containing 10 mM methyl-β-cyclodextrin (Mβ-CD) for 30 min at 37°C as we previously described (Wang *et al.*, 2004). After the cells were washed twice with serum-free medium, they were used for the experiments.

Analysis of Data

Data are given as the mean ± SE. Statistical analysis was performed using the Student's *t* test, and significance was accepted at $p < 0.05$. Each presented immunoblot is representative of the similar results of at least three separate experiments.

RESULTS

Enrichment of PLC-γ1 and IP3R2 in the Caveolin-associated Na/K-ATPase Preparation

The most widely used procedure for purification of Na/K-ATPase involves the treatment of crude membranes with relatively low concentrations of SDS that leave most of the Na/K-ATPase within the membrane, but solubilize and remove many impurities (Jorgensen, 1988). We used a modification of this procedure combined with glycerol gradient centrifugation (see *Materials and Methods*) to prepare two partially purified fractions (heavy and light) of the enzyme from rat kidney outer medulla and analyzed both fractions by Western blot for the presence of the signaling partners of Na/K-ATPase. The rat kidney was chosen for this study because the rat protein database is available for proteomic analysis. As shown in Figure 1, both α1 and β1 subunits were coenriched in the heavy fraction as expected (Jorgensen, 1988). When Na/K-ATPase activity was measured, a sevenfold increase in the specific activity was noted in comparison to that of the microsomal preparation. However, there was little coenrichment of Src and caveolin-1 (Figure 1). In contrast, when the light fraction was analyzed, we found that Src and caveolin-1 were coenriched with the Na/K-ATPase. In addition, the soluble ERKs were also modestly enriched in this light fraction. These findings led us to speculate that this caveolin-enriched light fraction may contain most of the signaling Na/K-ATPase and its partners. To further test this possibility and identify other unknown partners of the signaling Na/K-ATPase, we separated the light fraction on SDS-PAGE. After silver staining, four discrete bands with apparent molecular mass of the β1 subunit (60 kDa), the α1 subunit (100 kDa) of Na/K-ATPase, 150 kDa and 240 kDa, were excised and subjected to in gel trypsin-digestion. Subsequently, the masses of the resultant peptides were determined by MALDI-TOF analysis. As expected, data analysis identified the Na/K-ATPase β1 subunit from the 60-kDa band and the Na/K-ATPase α1 subunit from the 100-kDa band. We also identified Src kinase from the 60-kDa band (unpublished data), indicating that this method is sensitive for protein identification. MALDI-TOF analysis also revealed PLC-γ1 and IP3R2 from the 150- and the 240-kDa bands, respectively (Tables 1 and 2). These findings were confirmed by Western blot analysis (Figure 1). Like Src and caveolin-1, PLC-γ1 and IP3 receptors (isoforms 2 and 3) were also coenriched with the Na/K-ATPase (Figure 1), suggesting that these proteins could partner with the Na/K-ATPase to form a signalplex.

Different Domain of Na/K-ATPase Interacts with PLC-γ1 and IP3 Receptors

To test the hypothesis that both PLC-γ1 and IP3 receptors are partners of the signaling Na/K-ATPase, we first deter-

Table 2. MS-identification of PLC-γ1

m/z Submitted	MH^+ matched	Delta Da	Modifications	Start	End	Database sequence
905.3800	904.4066	0.97		824	830	(K) QDGGWWR (G)
905.3800	905.5209	−0.14		688	695	(R) DGAFLVRK (R)
909.9400	908.4478	1.5		198	204	(R) LTDFEQR (S)
1012.3100	1012.5111	−0.20	1Cys-am	602	609	(R) NGKVQHCR(I)
1083.1200	1083.4635	−0.34		1071	1079	(R) DEAFDPFDK (S)
1154.7200	1153.6482	1.1		997	1005	(K) GKKFLQYNR (L)
1313.9800	1312.6762	1.3		159	168	(R) KQFYSVDRNR (E)
1332.8400	1333.7163	−0.88	1Met-ox	188	197	(R) VPNMRFLRER(L)
1506.1800	1505.7872	0.39	1Cys-am	602	613	(R) NGKVQHCRIHSR(Q)

mined whether the α1 subunit of Na/K-ATPase possesses functional domains that can directly interact with PLC-γ1 and IP3 receptors. To do so, we expressed and purified GST-fused N-terminus (NT) and GST-fused central loop (CD3) of the Na/K-ATPase α1 subunit connecting the transmembrane helices 4 and 5 (Figure 2A) and then performed GST pulldown assays. Analysis of the pulldown proteins by Western blot revealed that PLC-γ1 binds to GST-CD3, but not to GST-NT and GST (Figure 2B). On the other hand, we found that IP3R2 interacts with GST-NT, but not with GST (Figure 2C). We also observed the interaction between the GST-NT and IP3R3, which was reported previously (Miyakawa-Naito *et al.*, 2003). In addition, it appears that there was also a weak interaction between IP3R3 and GST-CD3 (Figure 2C). Thus, these findings suggest that the Na/K-ATPase α1 subunit can function as a scaffold, capable of tethering PLC-γ1 and its effector IP3 receptors together to form a signalplex via different domains.

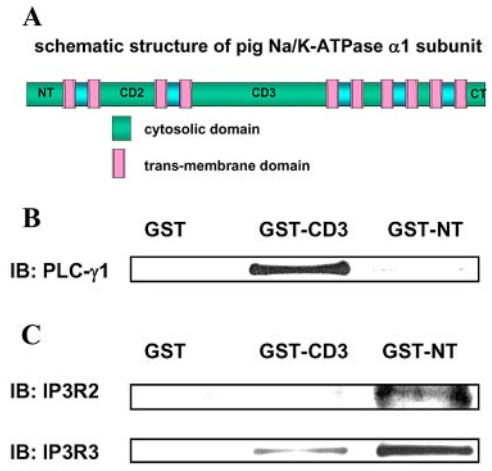

Figure 2. Binding of PLC-γ1 and IP3R2 to different domains of Na/K-ATPase α1 subunit. (A) Schematic structure of Na/K-ATPase α1 subunit is showed with the indication of cytosolic and transmembrane domains. GST-fused N-terminus (amino acid residue 6–90) and the GST-fused central loop (amino acid residue 350–785) of the Na/K-ATPase α1 subunit were prepared as described in *Materials and Methods*. NT, N-terminus; CT, C-terminus; CD3, cytosolic domain 3. (B and C) RIPA-solubilized LLC-PK1 cell lysates (1 mg) were incubated with the different GST fusion proteins, and a pulldown assay was performed. Western blot was carried out to analyze the pulldown proteins using specific antibodies as indicated.

Ouabain-activated Na/K-ATPase Signaling Complex Can Recruit and Activate PLC-γ1

Recently, we have demonstrated that ouabain activates the caveolar Na/K-ATPase signaling complex, resulting in tyrosine phosphorylation of multiple proteins and assembly of various signalplexes in LLC-PK1 cells (Wang et al., 2004). It is important to note that the term "ouabain activates" was used to describe the activation of the signaling function, but not the ion pumping function of the Na/K-ATPase. Because PLC-γ1 interacts with the α1 subunit of Na/K-ATPase (Figure 2B) and coenriched with the Na/K-ATPase in the light fraction prepared from rat kidney (Figure 1), we tested if the signaling Na/K-ATPase interacts with and regulates PLC-γ1 in LLC-PK1 cells. As depicted in Figure 3, A and B, LLC-PK1 cells were treated with 100 nM of ouabain for different times, and the cell lysates were immunoprecipitated with a polyclonal anti-Na/K-ATPase α1 antibody. Western blot analysis of the immunoprecipitates showed that PLC-γ1 was coprecipitated with the Na/K-ATPase α1 subunit in control LLC-PK1 cells, and ouabain significantly increased this interaction in a time-dependent manner. This ouabain effect was also dose-dependent. Significant changes were detected when the cells were exposed to 10 nM ouabain (Figure 3C). To corroborate the interaction, we repeated the time course experiments and immunoprecipitated the cell lysates with a monoclonal anti-PLC-γ1 antibody. Western blot analysis showed that ouabain increased the amount of coprecipitated Na/K-ATPase in a time-dependent manner (Figure 3D). We showed previously that Src activation and recruitment to the signaling Na/K-ATPase is essential for ouabain to evoke downstream cascades (Haas *et al.*, 2002). To address the role of Src in the ouabain-induced recruitment of PLC-γ1, we first probed for Src in the above immunoprecipitates, showing that ouabain stimulated the formation of the Na/K-ATPase/Src/PLC-γ1 complex (Figure 3D). These data suggest that the ouabain-induced recruitment of PLC-γ1 to the signaling Na/K-ATPase is likely due to the activation of Src. Therefore, in the second set of experiments, cells were pretreated with 1μM PP2, a Src inhibitor, and then exposed to ouabain. When cell lysates were immunoprecipitated with anti-Na/K-ATPase α1 antibody, we found that PP2 blocked the ouabain-induced increase in the amount of coprecipitated PLC-γ1 (Figure 3E). To further corroborate the role of Src, we repeated the above experiments in SYF cells that are derived from mouse embryos harboring functional null mutations in both alleles of the Src family kinases Src, Yes and Fyn. These experiments showed that ouabain failed to stimulate the interaction between Na/K-ATPase and PLC-γ1 (Figure 3F). On the other hand, ouabain was able to increase the interaction once Src is knocked back into the SYF cells (SYF+Src). Because mouse SYF and SYF+Src cells express

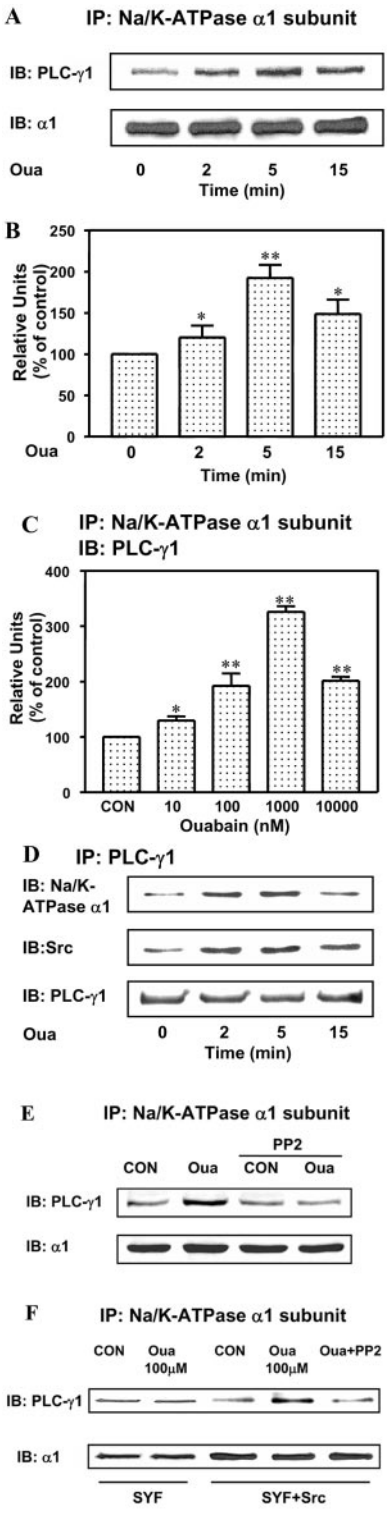

Figure 3. Effects of ouabain on interaction between the Na/K-ATPase and PLC-γ1. CON, control; Oua, ouabain treatment. (A–C) LLC-PK1 cells were treated with 100 nM of ouabain for different times (A and B) or with different concentrations of ouabain for 5 min (C). The cell lysates were immunoprecipitated with anti-Na/K-ATPase α1 antibody and then probed for coprecipitated PLC-γ1 by Western blot. (A) A representative Western blot and the quantitative data (mean ± SE) from four experiments are shown in B and C. Unless otherwise stated, the data are normalized to equal amounts of immunoprecipitated Na/K-ATPase α1. * $p < 0.05$; ** $p < 0.01$.

ouabain-insensitive Na/K-ATPase α1, 100 μM ouabain was used in these experiments (Figure 3F) as we previously reported (Haas *et al.*, 2002). These data indicate that activation of Src must make available additional binding sites for recruiting more PLC-γ1 to the Na/K-ATPase signaling complex.

Because we showed previously that ouabain-activated Na/K-ATPase/Src complex could transactivate EGFR (Haas *et al.*, 2000 and 2002), we reasoned that ouabain might activate PLC-γ1 via either the active Src or transactivated EGFR. To test this hypothesis, we first determined if ouabain stimulates tyrosine phosphorylation of PLC-γ1. LLC-PK1 cells were treated with 100 nM of ouabain for different times, and the cell lysates were immunoprecipitated with a monoclonal anti-PLC-γ1 antibody. Immunoprecipitates were then probed for active PLC-γ1 using a polyclonal antibody raised against Tyr^{783}-phosphorylated PLC-γ1. These experiments demonstrated that ouabain could activate PLC-γ1 in a time-dependent manner in LLC-PK1 cells (Figure 4, A and B). This result was confirmed when the cell lysates were immunoprecipitated by an anti-phosphotyrosine antibody, and then probed for PLC-γ1 (Figure 4, C and D). As expected, inhibition of Src by PP2 blocked the ouabain-induced tyrosine phosphorylation of PLC-γ1 (Figure 4, C and D). To further confirm that ouabain activates PLC-γ1, we measured the hydrolysis of PIP2 and the production of IP3 in LLC-PK1 cells in response to ouabain stimulation. To do so, we took the advantage of a newly developed assay based on a GFP-fused PLC-δ1 PH domain protein (Hirose *et al.*, 1999; Isshiki *et al.*, 2004). LLC-PK1 cells were transiently transfected with the expression vector. After 24 h, the cells were examined under confocal microscope. Consistent with the fact that the PH domain binds to PIP2, the expressed GFP-PH fusion protein appeared to be primarily associated with the plasma membrane in unstimulated cells (Figure 5A). Because the PH domain has at least equal binding affinity to IP3 compared with PIP2 (Hirose *et al.*, 1999), when PIP2 was hydrolyzed by PLC to produce IP3, the PH domain fusion protein would translocate into cytosolic compartments with IP3. This was demonstrated in control experiments in which the cells were treated with 10 μM ATP as previously reported (Isshiki *et al.*, 2004). When the cells were exposed to 100 nM of ouabain, the plasma membrane GFP signal decreased with a concomitant increase in cytosolic GFP signal, similar qualitatively to that observed after ATP treatment (Figure 5, B and C). This suggests that the ouabain-activated PLC-γ1 can catalyze the hydrolysis of PIP2. However, when compared with ATP, the ouabain-induced changes appeared to be much smaller than that of ATP (Figure 5).

Figure 3 (cont). (D) In D, cells were treated with 100 nM of ouabain for different times and the cell lysates were immunoprecipitated with anti-PLC-γ1 antibody. The immunoprecipitates were analyzed by Western blot as indicated, and the same experiments were repeated three times. (E and F) In E both control and PP2-pretreated LLC-PK1 cells (1 μM of PP2 for 15 min) were exposed to 100 nM of ouabain for 5 min. Cell lysates were immunoprecipitated with anti-Na/K-ATPase α1 antibody and then analyzed by Western blot using anti-PLC-γ1 and anti-Na/K-ATPase α1 antibody. In F, the same experiments as in E were conducted using either SYF or SYF+c-Src cells. Because these are ouabain-insensitive cells, 100 μM ouabain was used as we previously reported (Wang *et al.*, 2004). A representative Western blot is shown, and the same experiments were repeated three times.

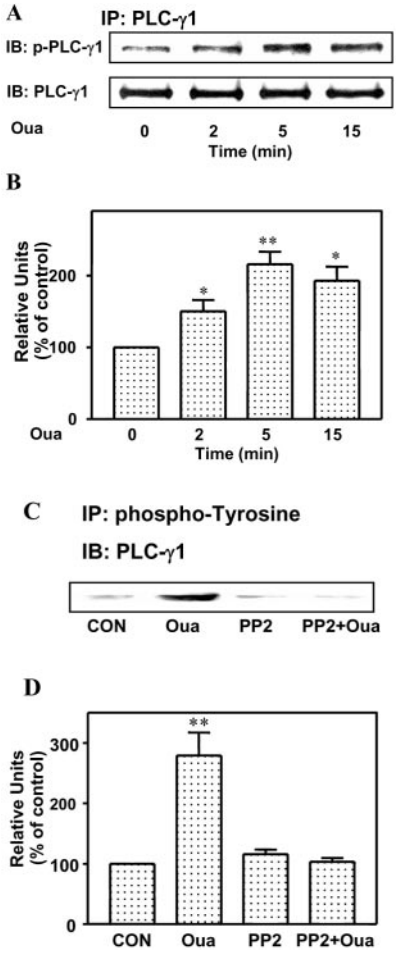

Figure 4. Activation of PLC-γ1 by ouabain. (A and B) Cells were treated with 100 nM of ouabain for different times. Cell lysates were immunoprecipitated with anti-PLC-γ1 antibody and then analyzed by Western blot using an antibody raised against the pY^{783}-PLC-γ1. A representative Western blot is shown in A, and quantitative data are mean ± SE of four independent experiments, shown in B. (C and D) Cells were pretreated with 1 μM PP2 for 15 min, and both control and PP2-treated cells were then exposed to 100 nM of ouabain for 5 min. Cells lysates were immunoprecipitated by anti-phosphotyrosine antibody and then probed for PLC-γ1. A representative Western blot is shown in C, and the quantitative data from three independent experiments (mean ± SE) are presented in D. ** $p < 0.01$.

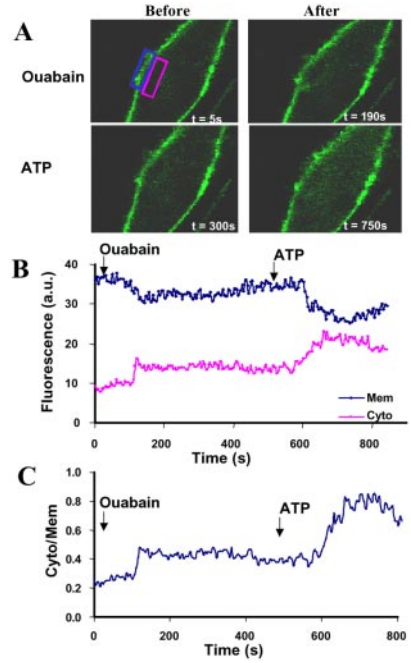

Figure 5. Effects of ouabain on PIP2 hydrolysis and IP3 production. Cells were transiently transfected with PLC-δ1 PH-GFP expression vector and then transfected cells were treated with 100 nM of ouabain, followed by 10 μM ATP. These cells were monitored for changes in cytosol and membrane GFP signal as described in *Materials and Methods*. (A) Confocal images of LLC-PK1 cells transiently expressing PLC-δ1 PH-GFP. Time shown at the bottom right corner of each images corresponds to the time point of the sequence shown in B. (B) The plasma membrane GFP signal (marked by a blue box) decreased with concomitant increase of cytosolic GFP signal (marked by a red box) in response to different stimuli as indicated. (C) The changes of cytosol/membrane GFP ratio in response to the stimuli. Similar findings were observed in total of 15 individual cells from five separate experiments.

Ouabain-activated Na/K-ATPase Signaling Complex Interacts with and Induces Tyrosine Phosphorylation of IP3 Receptors

There is evidence that the Na/K-ATPase interacts with IP3 receptors in kidney epithelial cells (Miyakawa-Naito *et al.*, 2003). The above data indicate that the ouabain-activated Na/K-ATPase signaling complex can recruit and activate PLC-γ1. Because the activated PLC-γ1 produces the ligand (IP3) of IP3 receptors, we tested whether the ouabain-activated complex could also recruit IP3 receptors in LLC-PK1 cells. Furthermore, we also tested whether the same ouabain-activated complex stimulates the tyrosine phosphorylation of IP3 receptors because Src family kinases can phosphorylate these receptors, resulting in increased sensitivity to IP3 (Jayaraman *et al.*, 1996; Yokoyama *et al.*, 2002; Cui *et al.*, 2004; Patterson *et al.*, 2004). As depicted in Figure 6A, we identified all three isoforms of IP3 receptors from LLC-PK1 cell lysates with commercially available antibodies. However, the anti-IP3R1 antibody produced a weak signal in comparison with other isoform-specific antibodies (Figure 6A). Control experiments also showed that the polyclonal anti-IP3R2 was a better choice for immunoprecipitation than the monoclonal anti-IP3R3. Therefore, the polyclonal antibody was used to immunoprecipitate IP3R in the following studies, whereas the monoclonal anti-IP3R3 antibody and the polyclonal anti-IP3R2 antibody were used to detect the coprecipitated IP3 receptors after immunoprecipitation with the polyclonal anti-Na/K-ATPase α1 antibody. As illustrated in Figure 6B, when cell lysates were immunoprecipitated by a polyclonal anti-Na/K-ATPase α1 antibody, we found that IP3R2 was coprecipitated. This interaction was regulated by ouabain in a time- and dose-dependent manner in LLC-PK1 cells (Figure 6, B to D). Significant increases were observed when cells were exposed to 10 nM of ouabain. In addition, when cell lysates were immunoprecipitated by anti-IP3R2 antibody and probed for the Na/K-ATPase α1 subunit, we confirmed that ouabain increased the interaction between the Na/K-ATPase and the IP3R2 (Figure 6E). Because different isoforms of the IP3 receptor can form hetero-tetrameric channels (Monkawa *et al.*, 1995; Miyakawa-Naito *et al.*, 2003), it was not surprising that all three isoforms were coprecipitated using either anti-Na/K-ATPase α1 or anti-IP3R2 antibody (unpublished data).

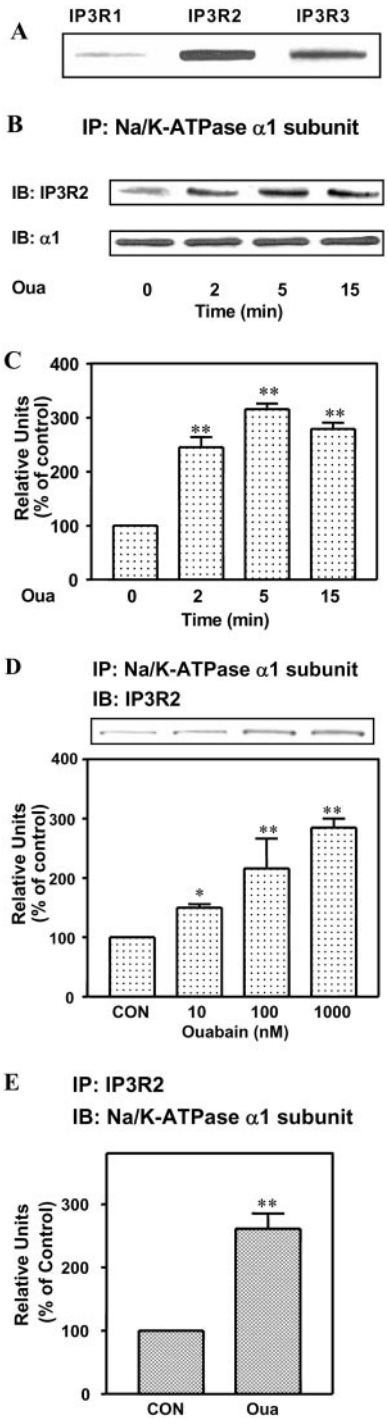

Figure 6. Effects of ouabain on interaction between Na/K-ATPase and IP3 receptors. (A) Detection of IP3 receptor isoforms from 40 μg of LLC-PK1 cell lysates with commercially available antibodies. (B and C) Cells were treated with 1 μM ouabain for different times, and cell lysates were immunoprecipitated with a polyclonal anti-Na/K-ATPase α1 antibody and then analyzed by Western blot using anti-IP3R2 antibody. A representative Western blot is shown in B, and C shows the quantitative data (mean $\pm$ SE) from four independent experiments. (D) Cells were treated with different concentrations of ouabain for 5 min and analyzed as in B. Data are mean $\pm$ SE of four independent experiments. (E) Cells were treated with 1 μM ouabain for 5 min, and cell lysates were immunoprecipitated by anti-IP3R2 antibody and then probed for coprecipitated Na/K-ATPase α1 subunit. The data are mean $\pm$ SE of three independent experiments. * $p < 0.05$; ** $p < 0.01$.

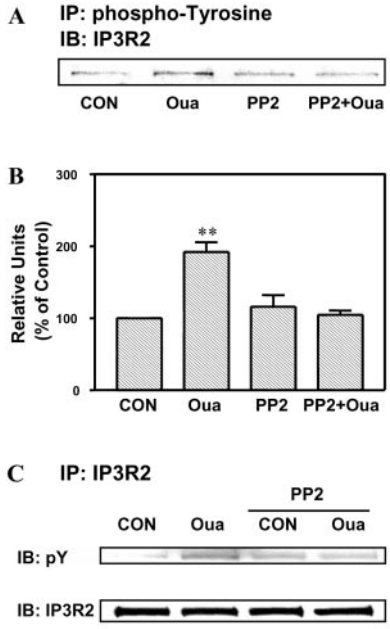

Figure 7. Effects of ouabain on tyrosine phosphorylation of IP3 receptors. (A and B) Cells were pretreated with 1 μM PP2 for 15 min, and both control and PP2-pretreated cells were then exposed to 100 nM of ouabain for 5 min. Cell lysates were immunoprecipitated with a polyclonal anti-phosphotyrosine antibody and then analyzed by Western blot using anti-IP3R2 antibody. (A) A representative Western blot and the quantitative data (mean $\pm$ SE) from three experiments are presented in B. ** $p < 0.01$. (C) Cells were treated as in A. Cell lysates were immunoprecipitated with anti-IP3R2 antibody and then analyzed by Western blot using a monoclonal anti-phosphotyrosine antibody. The same experiments were repeated four times. pY, phosphotyrosine

To test if ouabain stimulates the tyrosine phosphorylation of the IP3 receptors, LLC-PK1 cells were treated with 100 nM of ouabain for 5 min and cell lysates were immunoprecipitated with a polyclonal anti-phosphotyrosine antibody. As shown in Figures 7, A and B, ouabain increased tyrosine phosphorylation of the IP3R2. This was confirmed when the ouabain-treated cell lysates were immunoprecipitated by a polyclonal anti-IP3R2 antibody and analyzed by a monoclonal anti-phosphotyrosine antibody (Figure 7C). To test if ouabain-induced increases in tyrosine phosphorylation of IP3R2 are due to the activation of Src, we pretreated the cells with PP2 for 15 min and then exposed the cells to ouabain. As shown in Figure 7, A to C, inhibition of Src completely abolished ouabain-induced tyrosine phosphorylation of the IP3R2.

To confirm that Src mediates ouabain-induced tyrosine phosphorylation of IP3 receptors, we performed the following two sets of experiments. As depicted in Figure 8A, ouabain failed to stimulate tyrosine phosphorylation of the IP3R2 in SYF cells. However, ouabain was able to stimulate tyrosine phosphorylation of the IP3R2 once the cells were rescued by Src (SYF+Src cells). In the second set of experiments the IP3R2 was immunoprecipitated from LLC-PK1 cells by an anti-IP3R2 polyclonal antibody. After the immunoprecipitates were washed with PBS, we added either Mg^{2+}/ATP or recombinant active Src plus Mg^{2+}/ATP into the phosphorylation buffer. After 5-min incubation at 30°C, the reactions were stopped by the addition of 2× Laemmli buffer and analyzed for tyrosine phosphorylation by West-

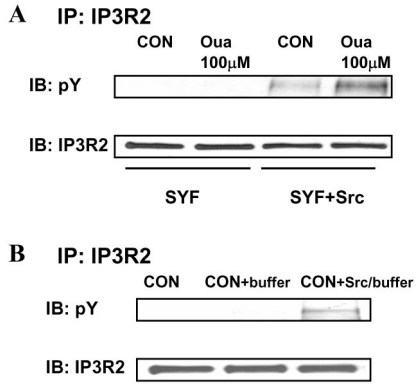

Figure 8. Src-mediated tyrosine phosphorylation of IP3 receptors. (A) Both SYF and SYF+Src cells were treated with 100 μM ouabain for 5 min as we previously reported (Wang *et al.,* 2004). Cell lysates were immunoprecipitated by anti-IP3R2 antibody and then probed for phosphotyrosine as in Figure 7C. The same experiments were repeated four times. (B) LLC-PK1 cell lysates were immunoprecipitated with anti-IP3R2 antibody, and the immunoprecipitates were washed in PBS. In vitro phosphorylation assay was performed as described in *Materials and Methods*. A representative Western blot is shown, and the same experiments were repeated three times.

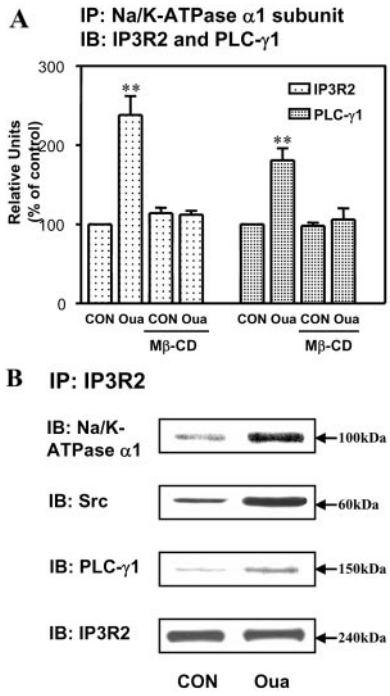

Figure 9. The signaling Na/K-ATPase tethers PLC-γ1 and IP3R2 together into a signalplex. (A) Cells were treated with methyl-β-cyclodextrin (Mβ-CD) as described in *Materials and Methods*. The control and cholesterol-depleted cells were then treated with 100 nM of ouabain for 5 min, and cell lysates were immunoprecipitated with anti-Na/K-ATPase α1 antibody. Western blot analysis of coprecipitated IP3R2 and PLC-γ1 was performed. The data are mean $\pm$ SE of three independent experiments. ** $p < 0.01$. (B) Cells were treated with 100 nM of ouabain for 5 min, and cell lysates were immunoprecipitated with anti-IP3R2 antibody. Western blot analysis of Na/K-ATPase α1 subunit, Src, and PLC-γ1 was performed. A representative Western blot is shown, and the same experiments were repeated three times.

ern blot. As illustrated in Figure 8B, addition of active Src in vitro was sufficient to stimulate the tyrosine phosphorylation of the IP3R2 in the presence of ATP. Taken together, these findings clearly demonstrated that the ouabain-activated Na/K-ATPase signaling complex could recruit and induce tyrosine phosphorylation of IP3R2 via Src in LLC-PK1 cells.

The Caveolar Na/K-ATPase Assembles the Complex in Response to Ouabain

We showed previously that caveolae played an essential role for the signaling Na/K-ATPase to interact with its partners (Wang *et al.,* 2004). To address the role of caveolae in ouabain-induced formation of the above signalplex, we treated LLC-PK1 cells with Mβ-CD to deplete cholesterol from plasma membrane. Depletion of cholesterol has been shown to reduce caveolar Na/K-ATPase and Src and to prevent the formation of the Na/K-ATPase/Src complex (Wang *et al.,* 2004). As depicted in Figure 9A, both control and Mβ-CD treated cells were exposed to 100 nM of ouabain for 5 min. Cell lysates were then immunoprecipitated with anti-Na/K-ATPase α1 antibody and probed for PLC-γ1 and IP3R2. As expected, depletion of cholesterol significantly reduced ouabain-induced increases in the interaction of Na/K-ATPase with both PLC-γ1 and IP3R2 (Figure 9A).

The Signaling Na/K-ATPase Tethers PLC-γ1 and IP3R2 Together into a Signalplex

The above findings indicate that the caveolar Na/K-ATPase may function as a scaffold, tethering PLC-γ1 and IP3R2 together, and thus facilitating the PLC-γ1-generated IP3 to act on IP3R2 in response to ouabain stimulation. However, because the immunoprecipitated Na/K-ATPase could contain two different pools that bind to either IP3R2 or PLC-γ1, we further examined this issue by immunoprecipitating the ouabain-treated cell lysates using an anti-IP3R2 antibody. As depicted in Figure 9B, the Na/K-ATPase, Src and PLC-γ1 were coprecipitated with IP3R2. Moreover, ouabain regulated these interactions (Figure 9B). Because ouabain-regulated interactions must involve the Na/K-ATPase, these data indicate that both PLC-γ1 and IP3R2 are brought together by the ouabain-activated Na/K-ATPase signaling complex.

Ouabain Stimulates Calcium Transients

To determine the functional role of the identified signalplex, we measured the effects of ouabain on $[Ca^{2+}]_i$ in LLC-PK1 cells. We found that ouabain-induced changes in $[Ca^{2+}]_i$ could be grouped into two types. Similar to the changes reported by Aperia's laboratory (Aizman et al., 2001), we found that ouabain could stimulate calcium oscillations (unpublished data). However, oscillatory signals occurred in <1% of the cells. In contrast, ouabain induced single calcium transient in ~40% of the LLC-PK1 cells. The effects occurred 2–4 min after ouabain exposure (Figure 10A) and at concentrations as low as 10 nM of ouabain (unpublished data). Significantly, in contrast to ouabain-induced calcium oscillations (Aizman *et al.,* 2001), ouabain elicited a single calcium transient even when the cells were incubated in Ca^{2+}-free medium (Figure 10B), indicating that ouabain could activate the release of Ca^{2+} from intracellular stores. Removal of extracellular Ca^{2+} did reduce the duration (1.79 $\pm$ 0.37 vs. 0.60 $\pm$ 0.05 min, $n = 54$, $p < 0.01$) of the Ca^{2+} transient, indicating that influx of Ca^{2+} is important for maintaining the transient. To test if the identified signalplex is in-

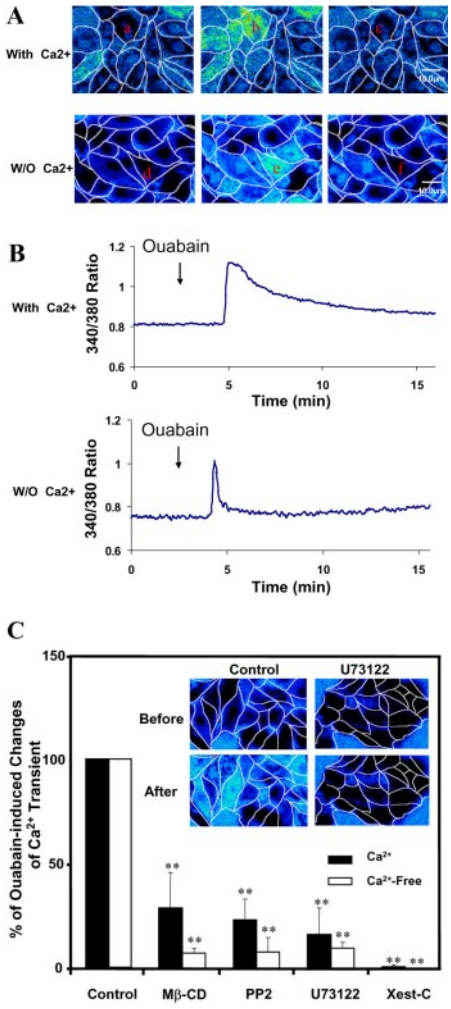

Figure 10. Effects of ouabain on $[Ca^{2+}]_i$. Cells were loaded with Fura-2/AM and changes in $[Ca^{2+}]_i$ were recorded as a function of time after the chamber was perfused with 100 nM of ouabain. (A) The actual images of the cells in response to ouabain stimulation in the presence (With) and absence (W/O) of extracellular calcium (a and d, control cells; b and e, the peak response after ouabain was added to the perfusate; c and f, 10 min after ouabain was added). (B) The ouabain-induced changes in $[Ca^{2+}]_i$ in a single cell as marked in A as a function of time. (C) The effects of different inhibitors on ouabain-induced calcium transients. Data are mean ± SE of at least 50 individual cells from 3 to 4 independent experiments, and the inset shows a representative recording of both control and the cells that were pretreated with PLC inhibitor, U73122. ** $p < 0.01$. Xest-C, Xestospongin C.

volved in the ouabain-induced activation of calcium release, we determined whether inhibition of PLC or IP3 receptor could reduce ouabain-induced calcium transients. Pretreatment of LLC-PK1 cells with inhibitors of either PLC (U73122, 2 μM) or IP3 receptor (Xestospongin C, 10 μM) blocked ouabain-induced calcium transients in both Ca^{2+}-free and regular Ca^{2+}-containing medium (Figure 10C). These data are consistent with the notion that ouabain stimulates the calcium release via the activation of PLC and the subsequent increase in intracellular IP3. This notion is further supported by the fact that inhibition of Src by PP2 or depletion of cholesterol by Mβ-CD also reduced the effect of ouabain on $[Ca^{2+}]_i$ (Figure 10C).

DISCUSSION

In previous work we demonstrated that the signaling Na/K-ATPase resided in caveolae in LLC-PK1 cells. Ouabain stimulated the Na/K-ATPase signaling complex, resulting in Src activation and subsequent tyrosine phosphorylation of multiple proteins and assembly of various signaling complexes (Wang *et al.*, 2004). In this report we presented evidence to show that the signaling Na/K-ATPase also possesses a scaffolding function, capable of tethering PLC-γ1 and IP3R2 into a signalplex via different domains. We also demonstrated that the ouabain-activated Na/K-ATPase signaling complex not only activated PLC-γ1, but also stimulated tyrosine phosphorylation of the IP3R2 via Src. These ouabain effects eventually resulted in Ca^{2+} transients in LLC-PK1 cells. Finally, we showed that an analysis of proteins coenriched with the Na/K-ATPase in combination with functional analysis might be a valuable tool for the identification of new interacting partners of the signaling Na/K-ATPase.

Na/K-ATPase as a Scaffold

There is compelling evidence that scaffolding function of proteins is essential for assembly and signal propagation of the signaling cascades. Classic examples include the assembly of the ERK cascade by the binding of adaptor proteins such as Shc and Grb to the tyrosine phosphorylated RTKs and AKAP-mediated coupling between protein kinases and their specific substrates (Lester and Scott, 1997; Pawson and Nash, 2000). Recent studies have demonstrated that membrane transporters such as NHE1 and band 3 anion channel can also function as a scaffold, tethering cytoskeleton and protein kinases together to form signalplexes (Tanner, 2002; Baumgartner *et al.*, 2004). Interestingly, disruption of the coupling activity of these membrane proteins affects many cellular signaling events (Tanner, 2002; Baumgartner *et al.*, 2004). The present study reveals that the signaling Na/K-ATPase also plays an important role in assembly of PLC-γ1 and IP3R2 into a signalplex. In vitro GST pulldown assays indicated that the central loop of the Na/K-ATPase α1 subunit interacts with PLC-γ1 (Figure 2B). On the other hand, the N-terminus of the Na/K-ATPase α1 subunit can bind the IP3 receptors (Figure 2C and Miyakawa-Naito *et al.*, 2003). These data suggest that the signaling Na/K-ATPase may be able to tether PLC-γ1 and IP3 receptors together via different scaffolding domains. This notion is supported by the following studies. First, both PLC-γ1 and IP3R2 coprecipitated with the signaling Na/K-ATPase in the control LLC-PK1 cells. Ouabain enhanced these interactions in a Src-dependent manner, suggesting that Src-induced tyrosine phosphorylation of either Na/K-ATPase or a component of the Na/K-ATPase signaling complex creates more binding sites for recruitment of these proteins to the complex. Needless to say, the identity of the binding sites remains to be addressed in the future studies. Second, immunoprecipitation of the IP3R2 coprecipitated both the Na/K-ATPase and PLC-γ1 (Figure 9B). Because ouabain regulated these interactions, it is most likely that the IP3R2 forms a tertiary complex with the Na/K-ATPase and PLC-γ1. Finally, because depletion of cholesterol inhibited ouabain-induced formation of the tertiary complex, it appears that caveolar Na/K-ATPase is involved in organization of this signalplex, which is consistent with our prior observation that the Na/K-ATPase transmits the ouabain signal from caveolae in LLC-PK1 cells (Wang *et al.*, 2004). This is also consistent with the findings presented in Figure 1, showing that both PLC-γ1 and IP3R2 were coenriched in the light fraction with the Na/K-ATPase,

caveolin-1 and Src when a modified Jorgensen method was used to isolate the Na/K-ATPase from the outer medulla of rat kidney. Remarkably, there is compelling evidence that IP3 receptors are in close proximity to the caveolar structure in many different cells including the renal epithelial cells (Fujimoto *et al.,* 1992; Bush *et al.,* 1994; Shaul and Anderson, 1998). Notably, the identified interaction in this report is reminiscent of the interaction between the caveolar Trp channels and the ER-localized IP3 receptors (Kiselyov *et al.,* 1999; Lockwich *et al.,* 2001). In addition, several studies have shown that caveolae not only contain PLC-γ1, but also 50% of the plasma membrane PIP2, a substrate of PLC-γ1 (Pike and Casey, 1996; Jang *et al.,* 2001).

PLC-γ1 catalyzes the conversion of PIP2 to DAG and IP3. Because IP3 acts on IP3 receptors to stimulate the release of calcium, we believe that the Na/K-ATPase accords proximity between PLC-γ1 and IP3R2, which would facilitate the conversion of extracellular ouabain signal into calcium transients. This notion is supported by the findings presented in Figures 9A and 10, showing that depletion of cholesterol or inhibition of Src prevented not only the formation of this signalplex, but also ouabain-induced calcium transients in LLC-PK1 cells. This notion is also consistent with a recent study showing that, although the activation of both muscarinic and bradykinin receptors stimulated the production of IP3, only bradykinin-mediated IP3 production induced calcium transient because this receptor is coupled to the IP3 receptors (Delmas *et al.,* 2002). Thus, the formation of signaling microdomains is of critical importance for the induction of selective and robust responses. In retrospect, realization that the Na/K-ATPase has scaffolding function should not be a surprise. There is ample evidence in the literature showing that the Na/K-ATPase is engaged in interactions with many cytoskeletal and membrane proteins (Nelson and Veshnock, 1987; Lee *et al.,* 2001; Jung *et al.,* 2004). For example, the interaction of the Na/K-ATPase with ankyrin and cofilin is well documented. However, our new findings are significant on several accounts. First, prior work has focused on how the Na/K-ATPase interactions with other proteins control the ion pumping function of the enzyme. We show here that the interactions between the Na/K-ATPase and its partners are important for the function of other interacting proteins and are essential for transmitting the extracellular ouabain signal. Second, these findings also illustrate the unique features of the Na/K-ATPase-mediated signal transduction. Unlike the classic scaffolding proteins such as Grb and AKAP, the Na/K-ATPase also has a borrowed tyrosine kinase activity when it forms a signaling complex with Src so that the activated complex can phosphorylate the effectors that bind to the scaffolding domains of the Na/K-ATPase (see next paragraph).

Regulation of PLC-γ1 and IP3R2 by the Activated Na/K-ATPase Signaling Complex

We reported previously that activation of Src is required for low concentrations of ouabain to increase $[Ca^{2+}]_i$ in cardiac myocytes (Tian *et al.,* 2001). Recent studies from other laboratories have also shown that ouabain evokes calcium oscillations in renal epithelial cells as well as in endothelial cells via pathways other than increases in intracellular Na^+ concentration (Aizman *et al.,* 2001; Saunders and Scheiner-Bobis, 2004). Specifically, Aperia's laboratory demonstrated that the signaling Na/K-ATPase formed a signaling complex with the IP3 receptors in renal epithelial cells and that ouabain regulated the interaction between the Na/K-ATPase and the IP3 receptors, resulting in calcium oscillations independent of activation of PLC and increase in IP3 (Miyakawa-Naito *et al.,* 2003). Their findings suggest that the ouabain-induced interaction between the Na/K-ATPase and the IP3 receptors may be sufficient to stimulate calcium oscillations (Miyakawa-Naito *et al.,* 2003).

When LLC-PK1 cells were exposed to ouabain, we observed two different types of change in $[Ca^{2+}]_i$. As reported (Aizman *et al.,* 2001), we found that ouabain indeed evoked low frequency calcium oscillations. However, this only occurred in <1% of the cells and made it difficult to study. On the other hand, we observed that ouabain stimulated calcium transients >40% of the cells (Figure 10). Because ouabain increased $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, we concluded that ouabain must stimulate the release of calcium via the activation of the signaling Na/K-ATPase. Indeed, as illustrated in Figures 3 and 4, ouabain activated PLC-γ1 in LLC-PK1 cells in a Src-dependent manner. Functionally, the activated PLC-γ1 was able to convert PIP2 to IP3 and DAG and inhibition of PLC abolished ouabain-induced calcium transients in LLC-PK1 cells (Figure 10C). This is consistent with our recent observation that ouabain-activated Na/K-ATPase stimulated PKCs in cardiac myocytes (Mohammadi *et al.,* 2001). In addition, we found that ouabain stimulated tyrosine phosphorylation of IP3R2 (Figures 7 and 8). This effect of ouabain is most likely due to the activation of Src. First, prior studies showed that Src family kinases could phosphorylate IP3R1 isoform (Jayaraman *et al.,* 1996; Yokoyama *et al.,* 2002; Cui *et al.,* 2004; Patterson *et al.,* 2004). Second, inhibition of Src abolished ouabain-induced tyrosine phosphorylation of the receptor (Figure 7). Third, ouabain could induce tyrosine phosphorylation of the receptor in Src-knock in, but not Src-knock out cells (Figure 8A). Finally, addition of purified and active Src to the immunoprecipitated IP3R2 induced tyrosine phosphorylation of the IP3 receptors (Figure 8B). These findings are interesting because recent studies have demonstrated that tyrosine phosphorylation of the IP3 receptors increases the sensitivity of the receptors to IP3 (Jayaraman *et al.,* 1996; Yokoyama *et al.,* 2002; Cui *et al.,* 2004; Patterson *et al.,* 2004). However, several issues remain to be resolved. First, it remains to be determined which isoform of the IP3 receptors is tyrosine-phosphorylated by the Na/K-ATPase signaling complex because all three isoforms are expressed in LLC-PK1 cells and can be coprecipitated under our experimental conditions. Second, it is not clear as to why only 40% of the cells showed significant changes in $[Ca^{2+}]_i$ in response to ouabain stimulation. Because ouabain only activated the caveolar Na/K-ATPase signaling complex (Figure 9A and Wang *et al.,* 2004), it is possible that Ca^{2+} changes in this discrete microdomain may not give rise to detectable changes at the whole cell level in most of the cells. Finally, it is important to note that 10 nM ouabain was sufficient to affect both PLC-γ1 and $[Ca^{2+}]_i$ in LLC-PK1 cells and that the effects were dose-dependent and correlated well with the established sensitivities of the α1 isoforms from different species (Figure 3, C and F, and Haas *et al.,* 2002). Because there is sufficient evidence that ouabain and marinobufagenin are likely the endogenous cardiotonic steroids, our findings brings about an interesting question as to whether this mode of regulation is relevant to in vivo physiology. To this end, several laboratories have reported that endogenous ouabain and marinobufagenin are circulated at sub-nM to nM concentrations in normal individuals and that volume expansion can significantly increase their concentrations (Hamlyn *et al.,* 1991; Fedorova *et al.,* 2002; Bauer *et al.,* 2005). Thus, it is conceivable that the described regulation may be relevant to in vivo physiopathology if future in vivo studies confirm our in vitro experiments.

In short, our new findings indicate that ouabain uses two separate pathways that may function synergistically in regulation of $[Ca^{2+}]_i$. First, the signaling Na/K-ATPase appeared to be able to force PLC-γ1 and IP3 receptors into the proximity to facilitate the signal transmission. This could be especially important for ouabain signaling because ouabain is a weak stimulus in induction of IP3 production in comparison to other stimuli such as ATP (Figure 5). Second, although ouabain activated PLC-γ1 and increased the production of IP3, it also stimulated the tyrosine phosphorylation of the IP3R2, which could sensitize the receptor to IP3. It is important to note that ouabain may regulate $[Ca^{2+}]_i$ via different modes of actions in a cell-specific manner. For example, whereas ouabain evokes calcium transients by assembling and activating the Na/K-ATPase/PLC-γ1/IP3R2 signalplex in LLC-PK1 cells, it may alternatively stimulate calcium oscillations by inducing the interaction between the Na/K-ATPase and IP3 receptors in primary culture of rat renal epithelial cells (Miyakawa-Naito *et al.*, 2003). Furthermore, in cardiac myocytes the ouabain-induced changes in $[Ca^{2+}]_i$ also involve the Na^+/Ca^{2+} exchanger.

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